PROLONGATION OF LIFE-SPAN OF SEA URCHIN SPERMATOZOA, AND IMPROVEMENT OF THE FERTILIZATION-REACTION, BY TREATMENT OF SPERMATOZOA AND EGGS WITH METAL-CHELATING AGENTS (AMINO ACIDS, VERSENE, DEDTC, OXINE, CUPRON)

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The addition of an amino acid to the sea water in which spermatozoa of sea urchins and other marine invertebrates are diluted has been found (Tyler, 1950; Tyler and Atkinson, 1950) to extend very considerably their functional life-span. By means of these agents the duration of fertilizable life and motility may be extended more than 50-fold. Treatment of the spermatozoa also has an effect on the nature of the fertilization reaction. Thus, eggs of Lytechinus often exhibit incomplete membrane elevation when fertilized by spermatozoa that have been diluted in sea water. In general this effect depends upon the extent of dilution and the age of the sperm suspensions. Treatment of the spermatozoa with an amino acid corrects this effect where it occurs with freshly diluted sperm and, parallel to the extension of fertilizable life and motility, extends the period during which a good fertilization-reaction is elicited. Runnström *et al.* (1946; *cf.*, Runnström, 1948; Wicklund and Gustafson, 1949) have reported that treatment of so-called underripe eggs of sea urchins with glycine and other amino acids and fertilization in these solutions improves their fertilizability and gives good membrane elevation. Tyler and Atkinson (1950) have also obtained this effect but note that pre-treatment of the eggs and inseminating in sea water gave much less improvement in fertilization and membrane elevation than obtained by treatment of the sperm.

In starfish another effect of amino acid treatment of the sperm was discovered by Metz and Donovan (1950). This consists in enabling the sperm to be agglutinated by the egg water of this species whereas ordinarily the agglutination reaction fails.

The extension of life-span of the sperm is accomplished with no apparent utilization of the amino acid. Thus determinations of glycine showed no significant loss during prolonged incubation with sperm, nor was there any significant production of ammonia (Tyler and Atkinson, 1950). Similar tests with carboxyl C^{14}-labelled glycine showed a negligible amount of decarboxylation (Tyler and Rothschild, 1951). The metabolism of the spermatozoa is, however, affected by the presence of the amino acid (Tyler and Rothschild, 1951). When sea urchin spermatozoa are diluted in ordinary sea water there is an initial great increase in rate of respiration followed by a decrease to barely measurable values accompanying loss of fertilizing

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capacity and motility. In the presence of amino acid the initial burst of oxygen uptake is suppressed, proportionately to the concentration of the amino acid. The rate then decreases to a relatively low but quite appreciable value where it remains rather constant during the extended life-span of the sperm. The total oxygen uptake of the treated sperm during its extended life span considerably exceeds that of the controls. From this, together with the evidence of non-utilization of the added amino acid it is clear that death of the sperm in ordinary sea water is not due to exhaustion of oxidizable substrate. The addition of amino acid evidently enables the spermatozoa to utilize more fully their endogenous substrate. Another interesting effect of the amino acid is to enable the spermatozoa to remain motile and retain fertilizing capacity under anaerobic conditions, whereas in ordinary sea water they die promptly in absence of oxygen.

In view of the above-mentioned evidence it was suggested (Tyler and Rothschild, 1951) that the amino acids act by virtue of their ability (*cf.*, Greenberg, 1951; Martell and Calvin, 1952) to bind certain heavy metals ordinarily present in sea water. This hypothesis is supported by the results of tests with other kinds of metal-chelating agents and with artificial sea water reported in the present paper.

Material and Methods

The animals used in these experiments were: the sea urchins *Arbacia punctulata, Strongylocentrotus purpuratus* and *Lytechinus pictus;* the sand dollar *Echinarachnius parma* and the polychaet *Chaetopterus pergamentaceus.* The eggs and sperm were obtained from the sea urchins and the sand dollars by the KCl-injection method (Tyler, 1949). From Chaetopterus they were obtained by excision of parapodia containing the ripe gonads.

The sperm concentrations are expressed as a percentage (1% and 0.1% in most of the experiments) of undiluted semen, the suspensions being prepared by direct dilution of the "dry" semen in the test solutions or immediately after a preliminary 5-fold dilution in sea water. "Dry" semen of the sea urchins *S. purpuratus* and *L. pictus* contains approximately 2×10^{10} spermatozoa per ml. The suspensions were kept at room temperature (19° to 22° C.) in Pyrex, 25 or 50 ml., stoppered Erlenmeyer flasks on a slow rocker (10 c.p.m. over an angle of 60°) or, in a few experiments, unshaken in a shallow (1 mm.) layer.

Fertilizing capacity was determined from the results of inseminating freshly collected eggs (ca. 500) in 4 ml. of sea water with various amounts (usually forming a 2-fold dilution series) of the sperm suspensions. The senescing experimental and control sperm suspensions are thus compared with each other, as well as with the freshly diluted semen, on the basis of the amount required to give the same percentage of fertilization, where this percentage is less than 100 and greater than 0. From this the relative duration of fertilizable life is obtained. Since fertilizing capacity tapers off gradually as the sperm suspensions approach the end of their life-span but shows a rather rapid decline around the 50 per cent value it was considered best to compare the suspensions on the basis of their half-life-spans. These are the figures given in Tables I and II. It should be noted, however, that on other bases of comparison such as "total" life-span or 10% life-span the results are qualitatively the same and the relative values do not differ very greatly.

For the motility determinations the intervals at which readings were taken

were determined on the basis of preliminary tests so that the usually sharp drop-off could be reasonably adequately covered. Motility was scored as 0, 1, 5, 25, 50, 75, 90 and 100 per cent. Again the duration of motility is expressed (Tables I and II) as a half-life. "Total" life-spans generally range some 50 to 100 per cent greater.

Sea water solutions of the various test substances were prepared from stock solutions made up with glass double-distilled water and adjusted to the pH of the sea water. Osmotic pressures were adjusted so that the most concentrated solutions employed did not deviate by more than 5 per cent from isotonicity with sea water. The sea water in most of the experiments was collected outside the laboratory at incoming tide and was filtered through washed "sharkskin" paper. It should be noted that it is important to check the pH of each solution made up by dilution of a stock solution in sea water. Thus, for example, when a stock solution of ethylenediaminetetraacetic acid at pH 8.2 is diluted with sea water to make a 10^{-3} molar solution there is a drop in pH to about 6.2. This is due to the displacement of protons from the weakly acidic ammonium groups of this compound as a result of chelation with some of the cations present in the sea water (*cf.*, Martell and Calvin, 1952).

Experiments

Life-span of spermatozoa in solutions of amino acids and peptides. In Table I the results of experiments with ten different amino acids and two peptides are summarized. All of these proved to be effective in extending both the fertilizable life and duration of motility of the spermatozoa. For glycine the optimum concentrations range around 0.05 and 0.1 molar. For the others the data are insufficient to specify optimum concentrations. The cysteine concentrations refer only to the initial solutions since this substance is rapidly oxidized to cystine upon preparation of the suspensions. The indications are that this may be effective in lower concentration than is glycine and the same may be true for glutathione.

The degree of extension of life-span obtained with these agents is dependent on the density of the sperm suspension. As is well known (*cf.*, Gray, 1928a, 1928b, 1931; Rothschild, 1948, 1951) the life-span of spermatozoa decreases with increasing dilution of the suspension. Comparison of the values of half-life-span in sea water (column headed 0) for the different semen concentrations, in experiments employing the same species, illustrates this dilution effect. In the amino acid solutions the "absolute" extension of life-span, in hours, is generally greater for the denser than the more dilute suspensions. The relative increase in life-span is, however, greater the more dilute the sperm suspension. Thus, in the experiments with Lytechinus listed in Table I the average half-life-spans for the 5, 1, 0.5 and 0.1 per cent sperm suspensions in 0.05 *M* glycine are, respectively, 7 to 12, 18 to 46, 50 to 80 and 100 times those for the sea water controls.

The present experiments also show a correlation between fertilizing capacity and motility. While it is well known (see Tyler, 1948; Rothschild, 1951 for references) that motile spermatozoa can be rendered non-fertilizing by various means, it is general experience that non-motile spermatozoa cannot effect fertilization. The present results indicate that loss of fertilizing capacity roughly parallels loss of motility.

Life-span of spermatozoa in solutions of other metal-chelating agents. In this category the effect of the following substances was investigated: ethylenediamine-

TABLE I

Action of amino acids on life-span of spermatozoa at 19°–22° C.

Substance	Species	Semen conc.	No. of experiments	Half-motile life* and half-fertilizable life† in: (concentration in sea water)						
				0	0.25 *M*	0.1 *M*	0.05 *M*	0.025 *M*	0.01 *M*	0.001 *M*
				hrs.	hrs.	hrs.	hrs.	hrs.	hrs.	hrs.
Glycine	*S. purp.*	1%	1*	2	48	53	27	7	5	1
		1%	1†	1	20	40	40	15	10	1
		0.1%	1*	0.5	26	31	17	2.5	0.5	1
		0.1%	1†	0.5	10	25	20	5	0.5	0.5
	L. pict.	5%	3*	1.5			18			
		5%	2†	2			15			
		1%	3*	1			18		1	1
		1%	1†	0.5			23			
		0.5%	8*	0.2			16			
		0.5%	3†	0.2			10			
		0.1%	2*	0.02	1.3	3	2		0.05	0.05
	A. punct.	1%	1*†	6–5			20			
		0.5%	1*†	2–1			49+			
		0.1%	1*†	0.5			4+			
		0.05%	2*†	<0.5			2.5+			
	E. parma	0.25%	1*†	1			8.5+			
	Ch. perg.	0.1%	1*†	0.3			4.5+			
Alanine	*L. pict.*	0.1%	1*†	0.02		1+				
	A. punct.	0.1%	1*†	1		24+				
Valine	*L. pict.*	0.1%	1*†	0.02		1+			0.02	
Leucine	*L. pict.*	0.1%	1*†	0.02		1+			0.02	
Lysine	*L. pict.*	0.1%	1*†	0.02		1+			0.02	
Glutamic	*L. pict.*	0.5%	1*	0.1			20+			
	A. punct.	0.1%	1*†	5			40+			
Histidine	*A. punct.*	0.5%	1*†	4			10+			
	A. punct.	0.1%	1*†	1.5			10+			
	A. punct.	0.01%	1*†	0.02			10+			
Phenylalanine	*A. punct.*	0.02%	1*†	0.02			3.5+			
	A. punct.	0.005%	1*†	0.02			3.5+			
Tryptophane	*A. punct.*	0.02%	1*†	0.02			3.5+			
	A. punct.	0.005%	1*†	0.02			3.5+			
Cysteine	*A. punct.*	0.05%	1*†	2			1		10+	10+
	A. punct.	0.01%	1*†	0.5			1		2	4
	A. punct.	0.002%	1*†	<0.02			0.5		2	2
Glycylglycine	*A. punct.*	0.5%	1*†	6			48+			
Glutathione	*L. pict.*	1%	1*	<0.4		3	2	2	1.4	0.5
	L. pict.	0.1%	1*	<0.3		1.8	2.5	1	1	0.3

tetraacetic acid (known commercially as Versene [2]), diethyldithiocarbamic acid (DEDTC), 8-hydroxyquinoline (oxine), α-benzoinoxime (cupron), N-nitroso-phenylhydroxylamine (cupferron), diazoaminobenzene, p-aminophenol, o-nitrophenol, 1–3, 4-trihydroxychalcone, p, p′-methylene bis N,N-dimethylaniline and 2-hydroxy-3-methoxybenzaldehyde. Only the first four of these were effective in

[2] I am indebted to the Bersworth Chemical Company, Framingham, Massachusetts, for samples of this substance; and to Dr. Linus Pauling for suggesting its use in connection with these experiments.

extending the life-span of the sperm. However, since the others were each tested in only one set of experiments there is the possibility that some of these might prove effective on further investigation.

The results of experiments with the first four substances are summarized in Table II. Of these, Versene and DEDTC have given the longest extensions of life-span. Versene proved to be highly effective at concentrations from 10^{-3} to 10^{-5} molar and DEDTC at concentrations of 10^{-3} and 10^{-4}. Two experiments not listed in the table also show DEDTC to be effective at 10^{-5} molar (7 hours half-motile life vs. 1½ hours for the sea water control with 1% Lytechinus sperm). Both substances were inhibitory at 10^{-2} molar.

TABLE II

Action of various metal-chelating agents on life-span of sea urchin spermatozoa at 19°–22° C.

Substance	Species	Semen conc.	No. of experiments	Half-motile life* and half-fertilizable life† in: (concentration in sea water)						
				0	10^{-2} *M*	10^{-3} *M*	10^{-4} *M*	10^{-5} *M*	10^{-6} *M*	10^{-7} *M*
				hrs.	hrs.	hrs.	hrs.	hrs.	hrs.	hrs.
Versene[1]	*S. purp.*	1%	8*	2.9	0.3	21	22	13	7	7
	S. purp.	1%	3†	1	<1	20+	20+	10–15		
	S. purp.	0.1%	4*	0.2	<0.1	3	10	1.5	0.3	0.2
	L. pict.	1%	4*	3.2		21	27	29		
	L. pict.	1%	2†	0.8		24+	24+	24+		
	L. pict.	0.1%	1*	0.2		18	22	13		
DEDTC[2]	*S. purp.*	1%	3*	5.6	2.5	17	5.5			
	L. pict.	1%	8*	5.8	2.7	22	32			
	L. pict.	1%	2†	1			30+			
	L. pict.	0.1%	1*	<0.5	1	4	2.5			
Oxine[3]	*S. purp.*	1%	3*	2		16	14	2		
	S. purp.	0.1%	1*	0.1		2	2	0.1		
	L. pict.	1%	2*	0.4		4.5		1.5	0.8	0.5
Cupron[4]	*S. purp.*	1%	2*	1			6	8.5	5	
	S. purp.	0.1%	2*	0.3			2	2	0.3	

[1] Ethylenediaminetetraacetic acid.
[2] Diethyldithiocarbamate.
[3] 8-Hydroxyquinoline.
[4] α-Benzoinoxime.

The degree of extension of life-span obtained with these agents is of the same order as was obtained by use of the amino acids. It seems reasonable to conclude, then, that they are acting in similar manner, namely by virtue of their metal-chelating capacity. Differences in effective range of concentrations can be attributed to differences in the dissociation constants of the metal-chelate compounds. Martell and Calvin (1952) have assembled data on the stability constants (reciprocal of dissociation constants) for an extensive series of metal-chelate compounds, including most of the agents used in this work. While the data on optimum concentrations accumulated here do not permit detailed quantitative comparisons, qualitatively the differences between the amino acid glycine and the other chelating agents, such as Versene, correspond to the differences in their relative avidity for metal ions.

Life-span of spermatozoa in artificial sea water with various amounts of calcium. In order to examine further the view that the extension of life-span by the amino acids and other metal-chelating agents was occasioned by the binding of certain metal ions, tests were made with artificial sea water. This was prepared from Merck Reagent Grade chemicals, of which the NaCl was especially low ($< 0.0001\%$) in heavy metals. Based on the sea water analyses of Lyman and Fleming (1940) the artificial sea water was made up with the following composition: 1000 ml. 0.55 *M* NaCl, 22 ml. 0.55 *M* KCl, 195 ml. 0.37 *M* $MgCl_2$, 103 ml. 0.37 *M* Na_2SO_4, 6 ml. 0.55 *M* $NaHCO_3$ and 35 ml. 0.37 *M* $CaCl_2$, adjusted to pH 8.2.

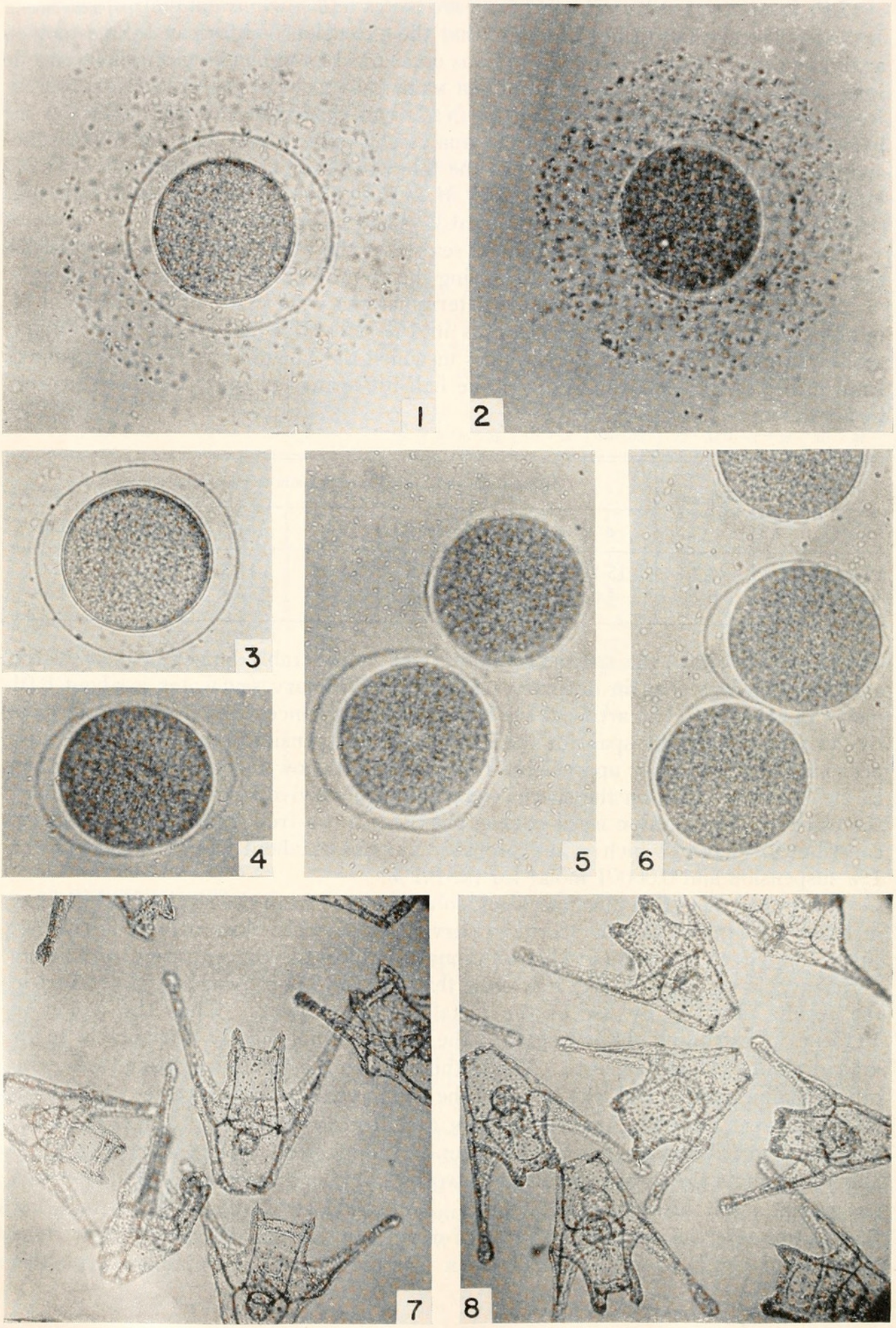
Along with the tests of the artificial sea water the effect of varying the concentration of calcium was also examined, using the above formula with various amounts of the $CaCl_2$ solution. Eight sets of determinations were made of the duration of motility of sperm of Strongylocentrotus in 1% and 0.1% suspension in ordinary sea water and in the artificial sea waters in which the calcium concentration ranged from 0 to 8×10^{-2} molar. The average half-life-spans, in hours, in these experiments were:

	Ordinary sea water	Artificial sea water containing calcium at molar concentration						
		0	0.0006	0.0025	0.01	0.02	0.04	0.08
1% sperm	3.7	15.1	14.6	15.3	16.1	11.3	12.4	8.2
0.1% sperm	0.7	3.6	4.1	3.3	6.7	0.8	1.6	1.4

As the figures show, the spermatozoa survived considerably longer in most of the artificial solutions than in ordinary sea water. Ordinary sea water is about 0.01 molar in calcium. The artificial sea water with this concentration of calcium gave the longest average life-span for the 0.1% sperm suspension but with the 1% suspension it did not differ appreciably from those with lower concentrations. Even in the "Ca-free" solution the sperm were found to survive longer than in ordinary sea water. This solution is, of course, not actually Ca-free since the added semen contributes calcium, which would probably amount to about 0.0001 molar for the 1% suspension and 0.00001 molar for the 0.1%.

It appears, then, that a balanced salt solution made from chemicals relatively low in heavy metals enables the sperm to survive longer than does natural sea water. Since this effect is obtained with solutions in which the concentration of calcium varies over a very wide range it is clear that this ion is not primarily concerned in the results obtained by use of the metal-chelating agents. Thus the action of Versene in extending the life-span of the sperm is not simply attributable to its known ability to bind calcium. While this chelating agent forms complexes with other alkaline earth metals the fact that the amino acids such as glycine, which form relatively weak complexes with these ions, are effective would tend to rule them out as being primarily involved in the life-span extending effect. It seems most reasonable to conclude that heavy metals are involved, although the present evidence does not permit ready identification of these. Rothschild and Tuft (1950) found that the dilution effect of sea water on rate of oxygen uptake could be imitated by trace amounts of $CuCl_2$ or $ZnCl_2$ dissolved in isotonic 'Analar' (containing negligible

PLATE I. Eggs and embryos of *Lytechinus pictus*. Magnifications: 175 × for Figures 1 to 6 and 65 × for Figures 7–8.



amounts of heavy metal) NaCl. The isotonic 'Analar' NaCl itself does not give a dilution effect when added to the sperm suspension. Glycine solutions can also abolish the dilution effect (Tyler and Rothschild, 1951). On the basis of this evidence it is suggested that Cu^{++} and Zn^{++} are among the heavy metal ions whose removal is responsible for the life-span extending effect of the chelating agents.

Effect of treatment of the sperm on membrane-elevation. As was mentioned earlier, treatment of the sperm with solutions of the amino acids improves the fertilization-reaction induced upon insemination with dilute and with aged sperm suspensions. The same effect is obtained with other chelating agents. In Plate I examples of good and poor membrane-elevation of eggs of Lytechinus are shown. The effect on the fertilization-reaction is illustrated by the results of the experiments with glycine and with Versene presented in Table 3. It may be noted that with sperm diluted in ordinary sea water, and aged only a short time, the type of reaction, along with the percentage fertilization, improves upon insemination with increasing amounts of sperm. However, with sperm that have been diluted in glycine or in Versene, and similarly aged, much smaller amounts suffice to give 100 per cent fertilization and normal membrane-elevation. Upon aging, the rapid drop-off in fertilizing capacity is seen along with the poor fertilization-reaction induced by the sea water-sperm. The glycine- or Versene-treated sperm, upon aging, maintain not only their fertilizing capacity but also their ability to induce good membrane-elevation. Towards the end of their extended life-span they, too, may induce poor membrane-elevation when used in amounts that were earlier effective in inducing a good reaction and 100 per cent fertilization. The type of reaction given by the eggs is evidently dependent upon the amount of sperm used for insemination as well as the age of the suspension. This holds both for the sea water-sperm and for the glycine- or Versene-treated sperm. Excessive amounts of sperm may sometimes be inhibitory, as the figures in Table VII indicate. Occasionally, also, sea water-sperm fail to give good membrane-elevation with any amount tested while the glycine- or Versene-treated sperm induce normal membrane-elevation.

It appears from these results that the condition of the inseminating sperm can determine the type of fertilization-reaction given by the eggs. As the spermatozoa age in ordinary sea water an increasing proportion of them become impaired in such a way that, while still capable of fertilization, they cannot elicit a normal response on the part of the egg. The relatively poorer response elicited by the smaller

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FIGURE 1. Good membrane-elevation shown by egg with intact jelly-coat inseminated with sperm treated with Versene (10^{-3} molar in sea-water, ½ hour).

FIGURE 2. Poor membrane-elevation shown by egg with intact jelly-coat inseminated with about 8 times as much sea water-treated sperm (note larger number of sperm in the jelly-coat than in that of Figure 1).

FIGURE 3. Good membrane-elevation shown by jellyless egg inseminated with Versene (10^{-3} *M*)-treated sperm.

FIGURE 4. Poor membrane-elevation shown by jellyless egg inseminated with very small amount of Versene (10^{-3} *M*)-treated sperm.

FIGURES 5 AND 6. Various types of poor membrane-elevation shown by Versene (10^{-3} *M*)-treated and sea water-washed eggs inseminated with sea water-treated sperm.

FIGURES 7 AND 8. Five day old plutei (not fed) from eggs fertilized and allowed to develop in sea water (Fig. 7) and in 10^{-3} molar Versene in sea water (Fig. 8).

amounts of inseminate can be interpreted on the basis of their greater dilution in the insemination dishes and consequent increase in proportion of impaired sperm in the interval before fertilization. The poor response given on occasion with excess sea water-sperm can be attributed to an increased probability of the impaired sperm, present in high proportion in such suspension, encountering the eggs; whereas with somewhat lower amounts of inseminate the more motile and presumably unimpaired sperm would have the advantage. Whether or not other factors, such as the liberation of antifertilizin from the sperm (see Tyler and Atkinson, 1950), might also be involved, would need to be further investigated.

TABLE III

Fertilization-reaction of eggs of Lytechinus inseminated with various amounts of sperm that have aged in sea water (s.w.), 0.05 M glycine (gl) and 0.001 M Versene (ve)

Age of 1% sperm susp.	Solution	Percentage of good membrane-elevation (G) and poor membrane-elevation (P) upon insemination of ca. 500 eggs in 4 ml. of sea water with following volumes of 1% sperm aged as indicated							
		0.05 ml.		0.10 ml.		0.2 ml.		0.4 ml.	
		G	P	G	P	G	P	G	P
(hours)									
0.02	s.w.	10	80	25	75	100	0	100	0
	gl.	100	0	100	0	100	0	100	0
0.5	s.w.	1	20	0	20	10	75	95	5
	gl.	100	0	98	0	100	0	100	0
1, 2, 5 and 10	s.w.	0	0	0	0	0	0	0	0
	gl.	100	0	100	0	100	0	100	0
18	s.w.	0	0	0	0	0	0	0	0
	gl.	90	5	100	0	95	5	100	0
23	s.w.	0	0	0	0	0	0	0	0
	gl.	0	15	10	30	35	50	85	15
0.2	s.w.	10	75	90	10	100	0	100	0
	ve.	100	0	100	0	90	10	100	10
1	s.w.	0.2	0	10	20	20	60	90	10
	ve.	100	0	100	0	100	0	100	0
2 and 4	s.w.	0	0	0	0	0	0	0	0
	ve.	100	0	100	0	100	0	100	0
10	s.w.	0	0	0	0	0	0	0	0
	ve.	15	3	95	5	100	0	100	0
21	s.w.	0	0	0	0	0	0	0	0
	ve.	30	5	45	10	85	5	100	0

Fertilization-reaction of Versene-treated eggs in Versene and in sea water. As was noted above (see introduction), treatment of sea urchin eggs with glycine, and fertilization in the solution, improves their fertilizability and type of reaction. This occurs also with other chelating agents. Table IV gives the results of some experiments with Versene. Comparison of the last three lines of the table with the first line shows the great improvement in percentage fertilization and in membrane-elevation that is obtained upon insemination in the Versene solutions. The sperm used in this experiment were freshly diluted in sea water but, of course,

TABLE IV

Fertilization-reaction of Versene-treated eggs of Lytechinus inseminated in sea water and in Versene

Eggs treated for ½ hour with	0.1 ml. of egg-suspension transferred to 4 ml. of	Percentage good membranes (G) and poor membranes (P) upon insemination with following volumes of 1% sperm			
		0.05 ml.		0.20 ml.	
		G	P	G	P
Sea water	Sea water	0	30	5	75
10^{-5} *M* Versene	Sea water	70	30	80	20
10^{-4} *M* Versene	Sea water	60	40	100	0
10^{-3} *M* Versene	Sea water	100	0	100	0
10^{-5} *M* Versene	10^{-5} *M* Versene	100	0	100	0
10^{-4} *M* Versene	10^{-4} *M* Versene	100	0	100	0
10^{-3} *M* Versene	10^{-3} *M* Versene	100	0	100	0

upon insemination of the eggs in the Versene solutions they are exposed to the action of this agent. So this type of experiment does not permit one to decide whether the effect is on the egg or sperm or both.

If the eggs are inseminated in sea water after treatment with Versene (lines 2, 3, and 4 of Table IV) there is much less improvement in the fertilization-reaction. In these tests insemination was done immediately after transfer to the sea water, and without further washing. It seemed likely that there might be sufficient carry-over of the Versene, which would perhaps diffuse only slowly from the gelatinous coat of the egg, to affect the results. Tests were therefore made of the effect of washing the eggs, after Versene-treatment, by repeated transfer in sea water or in Versene. The results of such a test are given in Table V. As the figures show, successive washing in sea water decreases the fertilizability of the Versene-treated eggs while those correspondingly transferred through Versene solution maintain their high fertilizability and ability to elevate good membranes.

Effect of removal of jelly-coat. It has been observed that the jelly-coat of eggs of various sea urchins swells considerably in solutions of amino acids and of proteins (Runnström *et al.*, 1943, 1946). This effect is readily observed, too, in Versene and DEDTC solutions. Thus, in 10^{-3} *M* Versene the jelly-coat of Lytechinus eggs swells within a half-hour to about double its original thickness.

TABLE V

Effect of washing in sea water on fertilization-reaction of Versene-treated eggs of Lytechinus

Eggs treated 2 hrs. in 10^{-3} *M* Versene and washed in:	Percentage good membranes (G) and poor membranes (P) upon insemination with 0.05 ml. of 1% sperm. Eggs washed by transferring 0.1 ml. to 4 ml. of new solution							
	First washing		Second washing		Third washing		Fourth washing	
	G	P	G	P	G	P	G	P
Sea water	25	75	0	50	0	30	0	5
10^{-3} *M* Versene	100	0	100	0	100	0	100	0

Concerning the action of the amino acids and proteins on the egg Runnström (1949, p. 251) points out that "This may facilitate penetration of spermatozoa and elevation of the fertilization membrane. But this is not the whole explanation of their improving effect." Eggs deprived of their jelly-coat when inseminated in these solutions also showed improved fertilization and membrane-elevation (*cf.*, Wicklund and Gustafson, 1949). It is suggested (Runnström, 1948) that these substances overcome a "cytoplasmic underripeness" of the egg. As the present work shows and was earlier reported (Tyler and Atkinson, 1950), treatment of the sperm alone with glycine, Versene, etc. induces good membrane-elevation. Treatment of the sperm with albumin (Wicklund, 1949) also has this effect. Also, increasing the amount of sea water-sperm used for insemination can result in improved membrane-elevation. It is not easy to see how a "cytoplasmic underripeness" can be involved in this.

In order to examine these questions further the reaction of jellyless eggs was investigated. The results of an experiment with Lytechinus eggs are given in

TABLE VI

Effect of removal of jelly-coat on fertilization-reaction of eggs of Lytechinus

Volume of freshly diluted 1% sperm used for insemination	Percentage good membranes (G) and poor membranes (P) upon insemination in sea water after similar washing in sea water:					
	I. Eggs from sea water suspension with intact jelly-coat		II. Eggs from (I) shaken to remove jelly-coat		III. Eggs treated ½ hour in 10^{-3} *M* Versene, shaken to remove jelly-coat	
	G	P	G	P	G	P
0.4 ml.	100	0	90	10	100	0
0.2 ml.	100	0	60	30	60	40
0.1 ml.	90	10	20	20	10	50
0.05 ml.	10	80	10	5	10	15
0.025 ml.	5	45	0.5	0.5	3	2
0.013 ml.	0	5	0.2	0.2	1	0.5

Table VI. The eggs had stood for ½ hour in 10^{-3} molar Versene (III) and in sea water (II) and were then shaken to remove their jelly-coat. That the jelly-coat was effectively removed was checked by microscopic examination. They were then washed in several changes of sea water and aliquots inseminated with various amounts of a freshly diluted sea water-sperm suspension along with aliquots of similarly washed sea water-eggs with intact jelly-coat (I). Comparison of II with III in this table shows that the pretreatment with Versene occasioned no significant improvement in the fertilization-reaction or in the percentage fertilization obtained with a given amount of sperm. The eggs with intact jelly-coat (I) showed better fertilization with corresponding amounts of sperm, and this is consistent with earlier experiments (Tyler, 1941; Runnström, 1947) along this line. Similar results have been obtained with eggs pretreated with glycine and inseminated in sea water after removal of the jelly-coat. Also pretreatment of jellyless eggs with glycine or with Versene and insemination in sea water did not improve fertilization in comparison with the sea water-exposed jellyless eggs.

Since the action of these agents does not persist when the eggs are returned to sea water it does not seem likely that a "cytoplasmic maturation," in whatever sense Runnström (1948) may mean this, can account for the results. On the other hand treatment of the sperm and insemination of jellyless eggs in sea water results in improved fertilization, as it does in eggs with intact jelly-coat (Table III). Three such experiments are listed in Table VII. The eggs were pretreated with Versene in order to facilitate removal of the jelly-coat, washed and inseminated in sea water with various amounts of sperm that had been diluted and aged briefly in 10^{-3} *M* Versene or in sea water. Comparison of the Versene-treated with the sea water-sperm shows the former to have some 8 to 16 times the fertilizing capacity of the latter, and to induce good membrane-elevation when amounts of sperm are

TABLE VII

Fertilization-reaction of jellyless eggs of Lytechinus inseminated with various amounts of Versene-treated and untreated sperm

Volume of 1% sperm used for insemination	Percentage good membranes (G) and poor membranes (P). Eggs treated for ½ to 1 hour in 10^{-3} *M* Versene, shaken to remove jelly-coat, transferred to sea water (4 ml.) and inseminated with indicated volumes of sperm at 10 to 15 minutes after dilution in:											
	(Experiment I)				(Experiment II)				(Experiment III)			
	Sea water		10^{-3} *M* Vers.		Sea water		10^{-3} *M* Vers.		Sea water		10^{-3} *M* Vers.	
	G	P	G	P	G	P	G	P	G	P	G	P
ml.												
0.8	80	20	100	0	0	100	100	0	0	100	100	0
0.4	100	0	100	0	20	80	100	0	5	95	100	0
0.2	95	5	90	10	60	30	80	20	60	40	100	0
0.1	40	60	99	1	20	20	100	0	10	50	40	60
0.05	5	90	97	3	10	5	100	0	10	15	90	10
0.025	5	15	99	1	0.5	0.5	98	2	3	2	60	40
0.0125	2	3	97	3	0.2	0.2	45	50	1	0.5	10	85
0.0063	0	0	20	40	0	0	20	5	0	0	30	20
0.0031	0	0	20	10	0	0	45	5	0	0	20	5
0.0016	0	0	8	2								
0.0008	0	0	0	0								

used that, in the case of the sea water controls, give mostly a poor fertilization-reaction. In both, the effect of quantity of sperm on type of reaction is shown, but with the Versene-treated sperm good membrane-elevation is given by considerably less sperm. This was illustrated also in the experiments on eggs with intact jelly-coat (Table III). An additional feature is observed in the results of the experiments of Table VII. With large amounts of sea water-sperm (0.8 ml. for experiment I; 0.8 and 0.4 ml. for experiments II and III) the fertilization-reaction is poorer than with the next lower amounts. This "optimum sperm concentration" effect is not regularly encountered in other experiments and presumably may be a property of particular sperm suspensions. An interpretation of this is offered in a preceding section. It may be concluded from the experiments with jellyless eggs that the improved fertilization-reaction induced in eggs with intact jelly-coat by

treated sperm is not primarily due to an effect of such sperm on the jelly-coat or to greater ease of penetration of the jelly-coat.

Development in glycine and in Versene solutions. Glycine, in rather low concentration, interferes with development. Thus in a test with Lytechinus eggs, fertilized and allowed to remain in 10^{-3} molar glycine, development did not proceed beyond the formation of abnormal gastrulae. In stronger solutions stereoblastulae were generally formed. While fertilization and cleavage are obtained in solutions as strong as 0.125 molar, development generally stops in the early blastula and disintegration soon sets in. Transfer to sea water soon after fertilization permits normal development, as Wicklund and Gustafson (1949) found with weaker solutions. In Versene solutions development of Lytechinus eggs was found to proceed quite normally in concentrations up to 0.001 molar. In one test, for example, eggs inseminated in 10^{-3}, 10^{-4} and 10^{-5} molar Versene gave 100 per cent fertilization and practically all eggs developed normally to the pluteus stage. Five-day old plutei from the 10^{-3} molar Versene solution and the sea water control are shown in Figures 7 and 8. Also eggs of Lychechinus that have been fertilized by sperm that have aged up to 24 hours in 10^{-3} molar Versene were found to develop normally to the pluteus stage.

Discussion

Most of the points raised by the present experiments have already been discussed above, but a few may be further emphasized here.

The ability of the metal-chelating agents, Versene, DEDTC, oxine and cupron, and of artificial sea water of low metal content, to extend the life-span of spermatozoa provides strong support for the previously expressed view (Tyler and Rothschild, 1951) that the trace metals normally present in sea water are responsible for the usual early death of the sperm in this medium. The life-span extending action of the amino acids and peptides (Tyler, 1950; Tyler and Atkinson, 1950) is explainable on this basis as is also the action of protein (Metz, 1945; Wicklund, 1949) and seminal fluid (Hayashi, 1945). In birds and mammals, amino acids, protein and seminal fluid may be similarly involved (Lorenz and Tyler, 1951; Tyler and Tanabe, 1952; Chang, 1947, 1949).

Very likely seminal fluid owes its action largely to its proteins. This can help explain the well-known *Dilution Effect; i.e.,* the decrease in life span with increasing dilution of the semen in sea water or physiological salt solutions. Thus, in the denser suspensions more seminal fluid protein would be available to bind the heavy metals present and eliminate their toxic action. If this were the whole explanation of the dilution effect then in the presence of the proper concentration of metal-chelating agent the dilute suspensions should survive as long as the denser ones. While this has been approached in some experiments reported here, in most cases the dilute suspensions have not lasted as long. So, the question remains open as to what extent other factors (see Gray, 1928a, 1928b, 1931; Rothschild, 1951) may be involved. However, it is now clear (*cf.,* Tyler and Rothschild, 1951) that the early death of sperm diluted in ordinary sea water is not due to exhaustion of endogenous food reserves.

Identification of the particular heavy metals that may be involved in the toxic action of ordinary sea water on sperm is not readily feasible from the present re-

sults. As mentioned above there are good reasons for suspecting Cu^{++} and Zn^{++}, but it is quite possible that others may also be concerned.

Of special interest is the ability of spermatozoa treated with amino acids and other chelating agents to improve the fertilization-reaction of the eggs. It is evident that the type of response given by the egg is not simply dependent upon the condition of the egg itself. The spermatozoon does not act in all-or-none manner in the sense of operating, or failing to operate, a trigger mechanism. Dependent upon its own condition the spermatozoon can elicit good or poor membrane-elevation on the part of the egg. This is not simply a matter of the treated sperm being able to traverse, and perhaps soften, the jelly-coat of the egg more effectively, since the effect is manifest also with jellyless eggs (Table VII).

In regard to possible effects on the egg the present experiments tend to rule out any maturing action. While insemination in Versene or glycine solutions improves fertilization, it is clear that this can be largely due to the effect on the sperm. Since pretreatment of the eggs with Versene or glycine and subsequent washing, and insemination in sea water, give no marked improvement in fertilization it appears that if there is any effect on the egg it is readily reversed. These substances cannot be considered to overcome any supposed "cytoplasmic underripeness" (Runnström, 1948, 1949) of the egg.[3] There are evidently effects of these substances on the eggs as manifest by swelling of the jelly coat and apparently also changes in the egg cytoplasm or its surface (*cf.*, Runnström and Monné, 1945; Runnström, 1948). However if these changes have any action in the direction of improved fertilizability of the egg it is evident that such effect largely disappears upon return to ordinary sea water, and it could hardly be considered a "maturating" effect. One might attempt to assess a possible fertilization-favoring effect on the egg by comparison of the results of inseminating also with treated-sperm eggs in sea water and in a solution of chelating agent. However, it would be difficult to decide to what extent the results are influenced by the effect on the sperm of the two different media in the insemination dishes. Runnström and his coworkers have contributed much to our knowledge of fertilization. While their extensive studies on "underripe" and "overripe" eggs do not represent a major part of their contributions it would appear desirable to re-examine the application of these terms to eggs. When difficulties in fertilization are encountered it would be important in the first place to know to what extent this is due to some agent ordinarily present in the sea water. Certainly where treatment of the sperm alone can improve the fertilization-reaction it does not seem reasonable to assume an "underripe" or "overripe" condition of the egg.

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[3] While this manuscript was in preparation Dr. Hans Borei sent the author a copy of a note which he had prepared for publication in Experimental Cell Research. He reports that insemination in Versene solutions improves the fertilizability of underripe and overripe eggs of *Psammechinus miliaris*. Since pretreatment and similar tests were not performed he is undecided as to whether the increased fertilizability is due to effects on the eggs, the sperm, or both.

Summary

1. The duration of motility and of fertilizing capacity of sea urchin sperm diluted in sea water can be considerably extended by the addition of any one of certain metal-chelating agents.These include ethylenediaminetetraacetate (Versene), diethyldithiocarbamate, 8-hydroxyquinoline, α-benzoinoxime and various amino acids and peptides (glycine, alanine, valine, leucine, lysine, glutamic acid, histidine, phenylalanine, tryptophane, cysteine, glycylglycine, glutathione). The relative increase in life-span, in the presence of these agents, is greater the more dilute the sperm suspension, while the absolute increase is generally greater for the more concentrated suspensions. Over 100-fold extension of life-span of dilute suspensions has been obtained by use of these agents.

2. An artificial sea water of low heavy metal content also enables the sperm to survive longer than in ordinary sea water. This effect is obtained with artificial solutions in which the calcium concentration ranges from 8 times to 1/100 that of sea water.

3. The results support the previously suggested view that the increased survival of the sperm in presence of amino acids, proteins, etc. is due to the ability of these agents to bind heavy metals present in the dilution medium. It is suggested that Cu^{++} and Zn^{++} are among the metals involved.

4. The "Dilution Effect" (decreasing life-span with increasing dilution of suspension) is largely explained on the basis of similar action of the seminal fluid proteins which are present in higher concentration in the denser suspensions prepared by direct dilution of the semen.

5. Treatment of the sperm with glycine or with Versene improves the fertilization-reaction (membrane-elevation) induced upon insemination of eggs in sea water with a given amount and age of sperm suspension. This effect persists along with the increased survival of the sperm in these agents. The results demonstrate that the type of response given by the egg can be determined by the condition of the inseminating sperm. The spermatozoon evidently does not simply act in "all-or-none" manner, in the sense of operating a trigger mechanism in the egg.

6. Fertilization is also improved when the eggs are inseminated in the presence of Versene or glycine, but the effect is largely eliminated if the eggs are washed and inseminated in sea water. Swelling of the jelly-coat that occurs in these solutions is not an important factor in the results. Eggs that have been deprived of their jelly-coat behave similarly, showing improved fertilization in the solutions and no persistent pretreatment effect. Such eggs also show the improved response to treated sperm. It is concluded that if there is any effect of these agents on the egg in the direction of improving fertilizability such effect is readily reversed upon transfer to sea water and does not constitute a "maturating" effect.

7. Eggs of *L. pictus* and *S. purpuratus* develop normally to the pluteus stage in 10^{-3} molar Versene but not in 10^{-3} molar glycine.

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